© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev177188. doi:10.1242/dev.177188

REVIEW

Inner ear organoids: new tools to understand neurosensory cell development, degeneration and regeneration

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ABSTRACT

The development of therapeutic interventions for hearing loss requires fundamental knowledge about the signaling pathways controlling tissue development as well as the establishment of human cell-based assays to validate therapeutic strategies *ex vivo*. Recent advances in the field of stem cell biology and organoid culture systems allow the expansion and differentiation of tissue-specific progenitors and pluripotent stem cells *in vitro* into functional hair cells and otic-like neurons. We discuss how inner ear organoids have been developed and how they offer for the first time the opportunity to validate drug-based therapies, gene-targeting approaches and cell replacement strategies.

KEY WORDS: Inner ear, Hair cells, Organoids, Regeneration, Stem cells

Introduction

The inner ear harbors a population of specialized sensory cells, the so-called 'hair cells', capable of transducing mechanical stimulation into electrochemical signals. Sound perception relies on the function of hair cells located in the cochlea's sensory epithelium, also known as the organ of Corti, which in turn activate the auditory neurons of the spiral ganglion. Additional sensory patches in the vestibular compartment, consisting of the maculae in the utricle and saccule, and cristae of the semicircular canals, contain hair cells that activate the vestibular neurons. These cells are responsible for perception of linear movement, gravity and head rotation that together contribute to the sense of balance. The sensory epithelia comprise a mosaic of cell types, including different classes of hair cells and supporting cells that position the hair cells and provide both cell-cell-mediated and soluble signals for their specialized function. Two types of hair cells are present in the cochlear sensory epithelium, inner hair cells (IHCs), which act as primary sound receptors, and outer hair cells (OHCs), which act to amplify sound-induced vibration in the epithelium (Fettiplace and Hackney, 2006; Géléoc and Holt, 2003). One row of IHCs and three rows of OHCs are intercalated by supporting cells in the cochlea. Vestibular hair cells are instead organized in patches overlaying the supporting cells and are further classified as Type I and Type II hair cells, based on their afferent innervation patterns, and electrophysiological and morphological features (Burns and Stone, 2017). A common characteristic of all hair cells is the presence of specialized stereocilia, organized in bundles, equipped with mechanically gated channels, known as mechanoelectrical transduction (MET) channels. Fluid vibration in the inner ear, caused by sound pressure waves or movement, displaces the stereocilia and results in the opening of MET channels, cellular depolarization and release of neurotransmitters that, in turn, activate the sensory neurons (Fettiplace and Hackney, 2006).

Hair cells and sensory neurons are vulnerable cell types, affected by noise overexposure and infections, as well as exposure to some classes of antibiotics and chemotherapeutics. Moreover, genetic factors play a central role in disease, and more than 100 genetic loci have been linked to non-syndromic deafness. For example, damage to the stereocilia by mechanical overstimulation by noise overexposure or loss of synaptic connectivity eventually results in sensory cell loss (Kujawa and Liberman, 2019). Uptake of ototoxic antibiotics, such as aminoglycosides (Huth et al., 2011; O'Sullivan et al., 2017), through the MET channels also results in neurosensory hearing loss. In mammals, loss of sensory cells is irreversible and leads to hearing impairment and balance problems, because the inner ear lacks an effective proliferative and regenerative capacity. Disabling hearing loss affects the quality of life of 460 million people worldwide (WHO 2019 factsheets; https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss) and, despite the scale of the problem, treatment options are limited. For comprehensive reviews on these topics, we refer the reader to Brown et al. (2008) and Müller and Barr-Gillespie (2015). New stem cell and gene therapies are being developed alongside pharmacological treatments, but their success strongly depends on the development of tools to validate therapeutic strategies *in vitro*.

The increasing awareness that three-dimensional (3D) cultures provide a more physiological environment for *ex vivo* tissue development has defined novel culture conditions for otic cells, so-called 'inner ear organoids'. Organoids consist of 3D cultures derived by differentiation of stem cells or tissue-specific progenitors, which recapitulate some of the original aspects of tissue organization, cellular composition and function of an organ. For the first time, inner ear organoids offer the possibility of studying sensory cell types of human origin *in vitro*. In this Review, we describe the development of these cell culture methodologies from tissue-specific and pluripotent stem cells (PSCs). We specifically refer to 'cochlear organoids' when generated from tissue-specific progenitors from the cochlea, or 'PSC-derived inner ear organoids' when derived from PSCs. We discuss potential applications, advantages and disadvantages, and their use as 'tools' for the development of therapeutic strategies for sensorineural hearing loss and other sensory deficits. In addition, we briefly review how knowledge on inner ear development and the analysis of the pathways that control cellular specification in the mammalian sensory organs, including tissue regeneration in newborn mammals and in non-mammalian vertebrates, has led to the identification of putative therapeutic targets. Given previous in-depth reviews on these topics

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(Atkinson et al., 2015), we focus on two specific aspects. First, how the modulation of these same pathways has allowed selective *ex vivo* expansion of tissue-specific progenitors from the postnatal murine cochlea and human fetal cochlea. Second, how faithful recapitulation of early stages of otic development *in vitro* has led to the robust generation of sensory hair cells and otic neurons from mouse and human PSCs.

Inner ear development and tissue regeneration

Mammalian inner ear development

The inner ear develops from the otic placode, which forms in the anterior portion of the embryo from pre-placodal ectoderm (PPE) (Kwon et al., 2010; Steventon et al., 2014; Streit, 2004). The PPE is a thickening of non-neural ectoderm (NNE) at the border between the neural tube and the surface ectoderm, which arises under the influence of a BMP gradient (Barth et al., 1999; Wilson and Hemmati-Brivanlou, 1995). During development, the otic placode invaginates and pinches off from the surface ectoderm to give rise to the otocyst (also known as the otic vesicle), which is induced by FGF and Wnt signals that are released by the otic mesenchyme and neural tube (Freter et al., 2008; Martin and Groves, 2006; Ohyama et al., 2007, 2006) (Fig. 1A). Upregulation of basic helix loop helix (bHLH) proneural transcription factors, such as neurogenin 1 and Neurod1, in a subpopulation of Sox2-positive cells in the otocyst leads to commitment of neuronal progenitors, which then delaminate from the otocyst and start to form the cochlear-vestibular ganglion (Appler and Goodrich, 2011; Evsen et al., 2013). Through proliferative events, remodeling and apoptosis, the otic vesicle gives rise to the remaining components of the inner ear, including sensory and non-sensory portions (Alsina and Whitfield, 2017; Basch et al., 2016a; Kelly and Chen, 2009). Six sensory epithelial patches form in the mammalian inner ear: the vestibular maculae of the utricle and saccule, the three cristae of the semicircular canals and the sensory epithelium in the cochlear duct (Fig. 1A,B).

Sox2 is one of the earliest markers of the prosensory domain, the region containing cells that are specified to become either sensory hair cells or supporting cells (Dabdoub et al., 2008; Kiernan et al., 2005b, 2006). In the absence of Sox2, neither cell type develops (Kiernan et al., 2005b). The prosensory domain also expresses the Notch ligand jagged 1 (Jag1) (Brooker et al., 2006; Kiernan et al., 2005a, 2006). In the cochlear duct, Jag1 expression becomes restricted to a population of cells on the neural side of the developing prosensory domain, whereas BMP4 is expressed on the abneural side (Ohyama et al., 2010). Gradients of Notch, BMP and FGF signaling across the prosensory domain contribute to the positioning and specification of the cells within the sensory epithelium (Basch et al., 2016a,b). Sox2-positive cells start to express the bHLH transcription factor Atoh1 before hair cell differentiation (Driver et al., 2013; Kelly et al., 2012; Pan et al., 2011; Woods et al., 2004). In the absence of Atoh1, hair cells fail to develop (Bermingham et al., 1999; Chen et al., 2002). The Notch ligands jagged 2 (Jag2) and delta-like 1 (Dll1) are expressed in the developing hair cells and induce Notch signaling in adjacent cells, which acts to repress Atoh1 expression in a process of lateral inhibition (Kiernan, 2013; Kiernan et al., 2005a; Lanford et al., 1999). This process leads to the generation of a mosaic of sensory hair cells and supporting cells (Kelley, 2006; Kelly et al., 2012) (Fig. 1C,D). Expression of the cell cycle inhibitor p27 (also known as Cdkn1b; Kip1) induces cell cycle exit in the developing cochlear sensory epithelium, starting at embryonic day (E)12.5-E13 of mouse development (Chen and Segil, 1999; Lee et al., 2006; Ruben, 1967) and week 7 to week 8 of human development (Roccio et al., 2018). Hair cell differentiation starts at the base of the cochlea at E16 in mouse and week 12 in humans (Chen and Segil, 1999; Locher et al., 2013; Roccio et al., 2018) (Fig. 1D,E).

Mammalian regeneration

The organ of Corti is postmitotic at birth and displays little regenerative capacity upon damage. This is in sharp contrast to the regenerative capacity observed in birds (Corwin and Cotanche, 1988; Ryals and Rubel, 1988), fish (Corwin, 1981) and amphibians (Corwin, 1985), in which, upon damage, supporting cells in the epithelia can replace lost hair cells either by trans-differentiation or mitotic regeneration (Atkinson et al., 2015; Monroe et al., 2015). Nonetheless, a number of studies have demonstrated some capacity for regeneration in vestibular (Burns et al., 2012; Burns and Stone, 2017; Forge et al., 1993; Lin et al., 2011; Warchol et al., 1993) and cochlear sensory epithelia of rodents (Bramhall et al., 2014; Cox et al., 2014; Hu et al., 2016), particularly in newborns. Indeed, two studies have recently shown that the sensory epithelium of the cochlea undergoes a limited extent of spontaneous regeneration after hair cell ablation during the first postnatal week (Bramhall et al., 2014; Cox et al., 2014). This has corroborated the hypothesis that supporting cells in the sensory epithelium can be triggered to replace or generate supernumerary hair cells (Bramhall et al., 2014; Cox et al., 2014; Hu et al., 2016; Jeon et al., 2011; Korrapati et al., 2013; Lowenheim et al., 1999; Mizutari et al., 2013; Shi et al., 2013; Walters et al., 2014).

Wnt signaling is required for the spontaneous regeneration of hair cells (Bramhall et al., 2014; Hu et al., 2016; Jansson et al., 2015). This activates supporting cells expressing the Wnt co-receptor and target, leucine rich repeat containing G protein coupled receptor 5 (Lgr5) (Chai et al., 2012; Shi et al., 2012; Wang et al., 2015). These studies also show a role for Wnt in regulating the expression of Atoh1 (Shi et al., 2010) and confirm the role of Notch and Sox2 in cochlear regeneration (Bramhall et al., 2014; Jeon et al., 2011; Kempfle et al., 2016; Li et al., 2015; Mizutari et al., 2013; Samarajeewa et al., 2018).

Probing the molecular pathways that underlie the regeneration of hair cells in these systems has proven valuable in advancing our understanding of the regenerative potential inherent to the neonatal cochlea. This limited regeneration-permissive time window precedes the final functional differentiation of hair cells and hearing onset, which occurs only after the first postnatal week in rodents (Appler and Goodrich, 2011).

The capacity for regeneration decreases in the adult and multiple mechanisms might account for this, including epigenetic silencing of key regulators and their targets or downregulation of the activity of key signaling pathways, such as Notch and Wnt, and transcription factors, such as Atoh1, involved in hair cell formation. *Atoh1* expression decreases during organ maturation, and cells in the cochlear sensory epithelium respond to Atoh1 induction by differentiating into hair cells only within a limited time window (Basch et al., 2016a,b; Costa et al., 2017; Kelly et al., 2012). This drop in regenerative potential could be due to epigenetic changes at the *Atoh1* locus (Stojanova et al., 2016), reduced chromatin accessibility of Atoh1 transcriptional targets (Jen et al., 2019; Stojanova et al., 2016) or the lack of factors that might cooperate with Atoh1 to promote hair cell differentiation, such as Gfi1 and Pou4f3 (also known as Brn3c) (Costa et al., 2017) or Isl1 (Yamashita et al., 2018).

As experimental strategies for developing hearing loss therapeutics have been reviewed in detail elsewhere (Géléoc and

Development (2019) 146, dev177188. doi:10.1242/dev.177188

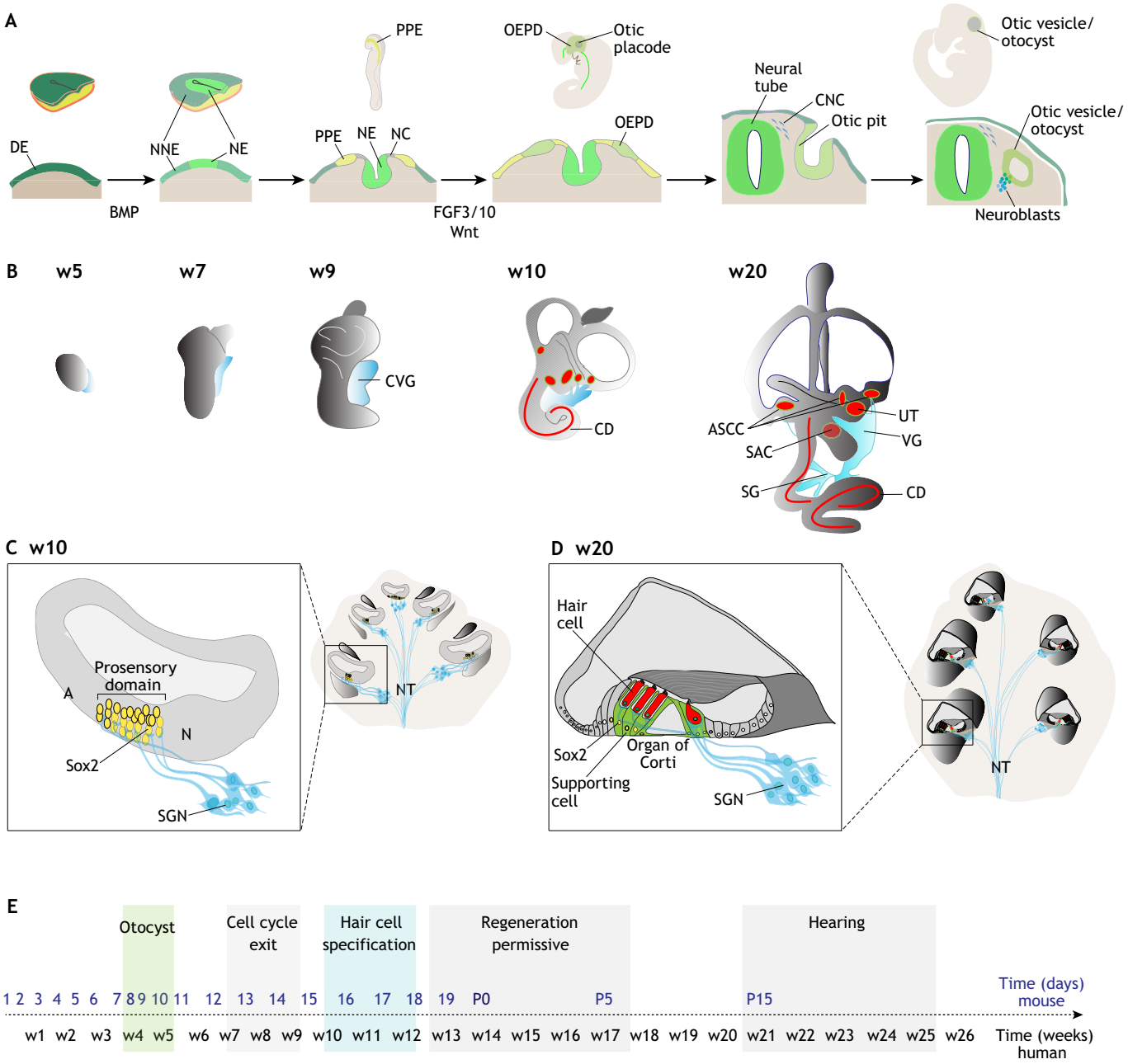

Fig. 1. Schematic of inner ear development. (A) Schematic of embryo development and corresponding tissue section, in the cranial portion, to illustrate otic development. Of the three embryonic germ layers, definitive ectoderm (DE) commits to neural fate, giving rise to neural ectoderm (NE). Non-neural ectoderm (NNE) is specified by a lateral-to-medial gradient of BMP signaling. Transient exposure to BMP signaling induces pre-placodal ectoderm (PPE) fate. All cranial placodes, including the otic epibranchial placode domain (OEPD), originate from the PPE. FGF and Wnt promote otic fate. The otic placode invaginates from the surface ectoderm to form the otic pit first, and then the otic vesicle or otocyst. Neuronal progenitors/neuroblasts (blue) delaminate from the otocyst and form the cochlear vestibular ganglion. NC, neural crest; CNC, cranial neural crest. (B) From week 4-5 of human fetal development (E9.5-10.5 mouse) the otocyst grows and gives rise to the components of the inner ear. Epithelial sensory patches are shown in red: three sensory cristae in the ampullae of the semicircular canals (ASCC), two sensory patches in the utricle (UT) and saccule (SAC), and the sensory epithelium in the cochlear duct (CD) contains mechanosensory hair cells. The developing cochlear vestibular ganglion (CVG) is depicted in blue. The vestibular ganglion (VG) neurons innervate the vestibular maculae and cristae. Spiral ganglion (SG) neurons innervate the CD. (C) Schematic of cochlear cross-sections at w10/E14 of development (left) and after maturation (postnatal day 15/w20) (right). The developing prosensory domain in the cochlea is marked by Sox2-positive cells (yellow). Spiral ganglion neurons (SGN) innervate the prosensory domain before hair cell maturation. A, abneural side; N, neural side; NT, nerve trunk. (D) The cochlear prosensory domains differentiate into the organ of Corti. Sensory hair cells are indicated in red, supporting cells in green. (E) Developmental timeline highlighting the steps associated with otocyst formation, cell cycle exit of the cochlear prosensory domain, specification, maturation and functionality of hair cells in the cochlear duct. Human timeline indicated in weeks (w) in black, mouse timeline in days in blue. Postnatal days (P) 0, 5 and 15 are indicated.

Holt, 2014; Müller and Barr-Gillespie, 2015) (Box 1), we focus here on the recent advancement in the field of inner ear organoids. We discuss how organoid tools could be exploited to develop novel therapeutic strategies for inner ear pathologies, including possibly gaining a better understanding of the regenerative potential of the human inner ear.

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Box 1. Therapeutic strategies to induce regeneration

Understanding the mechanisms that regulate regeneration in the neonatal sensory organs and prevent it in the adult is an area of extensive investigation. Therapeutic translations of these findings largely focus on re-activation of the same signaling pathways that control hair cell development or regulate regeneration in non-mammalian vertebrates, by chemical or genetic modification to 'boost' the regenerative capacity. Activating regeneration by inhibition of Notch activity has shown promising effects in neonatal conditions (Li et al., 2015; Maass et al., 2015; Mizutari et al., 2013). In the organ of Corti of adults, however, the activity of the Atoh1, Wnt and the Notch pathway is reduced, and the effects of Notch inhibition by GSI are consequently minimal compared with the neonatal situation (Basch et al., 2016b; Hartman et al., 2009; Maass et al., 2015). Moreover, responsiveness to GSI-treatment in the adult inner ear may depend on damage; expression of Notch downstream effectors increases after trauma, which causes overt hair cell loss (Batts et al., 2009; Du et al., 2018; Mizutari et al., 2013), but not after exposure to noise that induces less extensive hair cell death (Maass et al., 2015). Activation of canonical Wnt signaling though β-catenin stabilization also results in cell cycle re-entry of supporting cells (Li et al., 2015; Roccio et al., 2015; Samarajeewa et al., 2018) and prolongs the time window for GSI-induced hair cell differentiation. However, the effects decline after the first postnatal week. Although Wnt and Notch pathway components are expressed throughout early (P0) and late (P8) neonatal stages, targets related to cell proliferation and cell cycle progression are downregulated (Samarajeewa et al., 2018). Unlike the results in the newborn cochlea and vestibular organs (Bramhall et al., 2014; Jeon et al., 2011; Lin et al., 2011; McLean et al., 2017; Yamamoto et al., 2006), drugs and siRNAs targeting Wnt and Notch signaling show limited effects in adult models of hearing loss, such as from noise damage (Du et al., 2018; Mizutari et al., 2013; Tona et al., 2014). The generation of new hair cells through Atoh1 overexpression in some animal studies have demonstrated promise for the recovery of hearing (Izumikawa et al., 2005), although others have shown only marginal functional improvements (Atkinson et al., 2014).

Cochlear organoids from tissue-specific progenitors

Otic spheres from postnatal cochlear and vestibular cells

The establishment of neurosphere cultures has allowed the *in vitro* expansion of neural stem cells from the neurogenic niches of the rodent brain (Reynolds and Rietze, 2005; Reynolds and Weiss, 1996). These advances have led to the development of protocols to form 'otic spheres' (Malgrange et al., 2002; Oshima et al., 2007), which facilitate the isolation of putative stem or progenitor cells resident in the sensory tissues of the inner ear. Although variations in clonal origin, long-term self-renewal capacity and multi-potentiality of otic sphere-forming cells have not always been addressed, these types of assays allow detection of a pool of cells responding to mitogenic signals by re-entering the cell cycle. Indeed, epithelial progenitors can give rise *in vitro* to supporting cells or hair cells, whereas cell populations isolated from the spiral ganglion can differentiate into sensory neurons. Sphere forming efficiency and proliferative response, however, sharply decrease in the first few days after birth (Oshima et al., 2007). Lineage-tracing experiments and cell sorting have shown that glial cells within the ganglion could act as neuronal progenitors (Lang et al., 2015; McLean et al., 2016) and supporting cells represent hair cell progenitors of the inner ear sensory epithelia.

Additional cell sorting experiments have isolated supporting cells based on the expression of Sox2, p27, p75 (Ngfr), Lgr5 and others (Chai et al., 2012; Roccio et al., 2015; Shi et al., 2012; Sinkkonen et al., 2011; White et al., 2006). These experiments have revealed that, although several cell types in the cochlear epithelium can respond to mitogenic stimulation, the capacity to give rise to hair cells is limited to a subset of the supporting cells. Lgr5 has been proposed as one of the most stringent markers for the isolation of supporting cells with hair cell progenitor characteristics in the murine cochlea (Chai et al., 2012; Shi et al., 2013, 2012). The Lgr5-positive cells show a spontaneous response to damage in the newborn cochlea, where they proliferate and give rise to hair cells (Bramhall et al., 2014). This can be further enhanced by stimulation of Wnt signaling, Sox2 manipulation or Notch manipulation (Bramhall et al., 2014; Chai et al., 2012; Roccio et al., 2015; Shi et al., 2013, 2012). However, these properties are not exclusive to cells that express high levels of Lgr5 in the native cochlea, and recruitment of supporting cells that do not express Lgr5 has been observed after damage. Indeed, in the utricle, damage has been shown to increase both Lgr5 expression and regeneration of hair cells (Wang et al., 2015). Lgr5-positive supporting cells in the inner ear sensory epithelia appear, therefore, to acquire 'progenitor' identity under these experimental conditions.

Cochlear organoids from the murine sensory epithelium

Lgr5 was initially identified in the small intestine as a target of Wnt signaling. Cells in the crypt domains of the small intestine and colon that express Lgr5 were shown to be Wnt-responsive stem cells in this organ (Barker et al., 2007). Lgr5-positive cells isolated from the intestinal crypt domains could be expanded *in vitro* using culture conditions that allow for proliferation and final differentiation of all intestinal cell types with a morphological, functional and anatomical organization that recapitulates the original organ, and were therefore named 'gut organoids' or 'mini-gut' (Sato et al., 2009). Since this original organoid report, an increasing number of tissue-specific stem cells or progenitor cells from a variety of organs have successfully been expanded and differentiated *in vitro* using similar conditions (Barker et al., 2010; Huch et al., 2013; Sato et al., 2011). The use of Matrigel or extracellular matrix (ECM) proteins as scaffolds favors the assembly of epithelial cells in a configuration that recapitulates basal-apical polarity, tissue stiffness and cell-cell interactions more accurately than floating spheroid cultures. Identifying the signaling molecules that control stem cell proliferation and differentiation in these organs has recently allowed the refinement of protocols to achieve either cellular expansion or selective differentiation using growth factor- and small molecule-guidance (Yin et al., 2014).

Having shown the importance of Wnt signaling for the proliferation and differentiation of otic progenitor cells, and identified the Lgr5-expressing hair cell progenitors, we decided to translate the gut organoid generation methods to the inner ear sensory epithelia (McLean et al., 2017). We have demonstrated that the addition of Matrigel to cultures of cochlear sensory epithelium-derived dissociated cells gives rise to epithelial cysts, rather than spheres. In addition to growth factor stimulation (Oshima et al., 2007) and activation of Wnt signaling using CHIR99021 (Roccio et al., 2015), the modulation of chromatin remodeling by the histone deacetylase inhibitor valproic acid (Stockhausen et al., 2005) allows for robust expansion of Lgr5-positive supporting cells in these 3D cultures. Although the starting cell population contains only a small percentage of Lgr5-positive cells, exposure to these treatments upregulates Lgr5 and drives their expansion. In a second step, Notch signaling is inhibited using the gamma secretase inhibitor (GSI) LY411575 and Wnt signaling is activated, which promotes the differentiation of the Lgr5-positive cells to hair cells (Fig. 2A,C). Hair cells derived by these methods bear apical stereocilia with a luminal orientation and, interestingly, express markers of either IHCs, such as the glutamate transporter vGlut3, or OHCs, such as the motor protein prestin, but not both. This could indicate that

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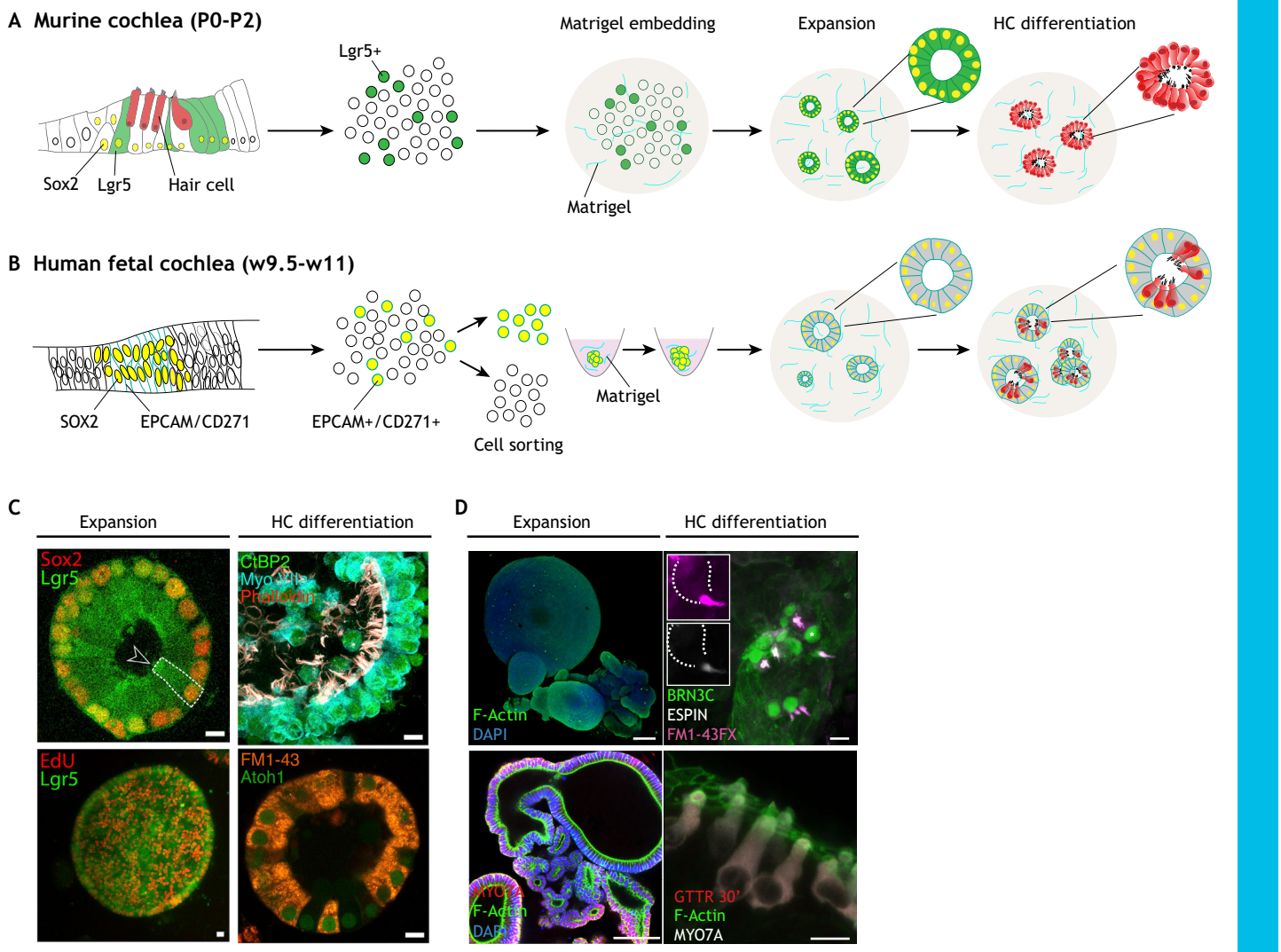

Fig. 2. Preparation of cochlear organoids from murine and human tissue-specific progenitors. (A) Organoid derivation from murine Lgr5-positive cochlear supporting cells, isolated from early postnatal mice (P0-P2) using an Lgr5-EGFP reporter line. The sensory epithelium is dissociated to a single cell suspension and embedded in Matrigel. A first phase of expansion of Lgr5-positive progenitors is followed by differentiation, induced by Wnt activation and Notch inhibition, resulting in conversion of Lgr5-positive supporting cells (green) to hair cells (HC; red). (B) Organoid derivation from human fetal prosensory domain cells. The whole cochlea is mechanically and enzymatically dissociated into a single cell suspension. EPCAM and CD271 (green) are used as surface markers to isolate Sox2-positive cells from the prosensory domain. Cells are sorted by flow cytometry and aggregated in round-bottom 96-well plates, then embedded in Matrigel and expanded for 2-3 weeks. Differentiation is induced in co-culture with the mesenchymal/neuronal progenitor pool and growth factor withdrawal for an additional 2-3 weeks. This results in organoids that contain supporting cells (gray) and hair cells (red). (C) Representative examples of cochlear organoids as in McLean et al. (2017). Immunostaining of the organoids during the expansion and differentiation phases. During expansion (left), epithelial cells in the cysts co-express Sox2 and Lgr5 (GFP) (upper panel) and are highly proliferative (lower panel). Upon differentiation (right), they express hair cell markers such as Myo7a, CtBP2 and F-actin-positive bundles (upper panel). Cells also express Atoh1 and are capable of taking up the FM1-43 dye, suggesting active MET channels (lower panel). The dashed line indicates the border of a single supporting cell. The arrowhead points at the apical surface, facing the lumen. (D) Representative examples of cochlear organoids from human fetal tissue as in Roccio et al. (2018). Immunostaining of the organoids during the expansion and differentiation phases. During the expansion phase (left), sorted cells grow as epithelial structures expressing different cochlear duct and prosensory domain markers and lack differentiation signs (MYO7A, F-actin bundles). After differentiation (right), patches of hair cells expressing BRN3C, espin and MYO7A are found. *In vitro*-generated hair cells show functional MET channels as detected by FM1-43 uptake (upper panel) and GTTR uptake (lower panel). The dashed line indicates the contour of a single hair cell. The asterisk marks the cell enlarged in the insets. Scale bars: 15 µm in C; 100 µm in D (expansion); 10 µm in D (differentiation).

different supporting cell types possess identity for the medial or lateral parts of the organ of Corti and act as progenitors for IHCs and OHCs, respectively – an intriguing possibility awaiting demonstration. Although these results have been exclusively validated in first-generation cultures, and cells have not been analyzed for their capacity to propagate after consecutive passaging, the yield and maturation of the *in vitro*-generated hair cells has proved superior to approaches that rely on withdrawal of growth factors in otic sphere culture (Shi et al., 2012). It is important to note that cochlear organoids can be generated by clonal expansion of Lgr5-positive cells more efficiently when cells are obtained from newborn mouse tissues than when obtained from the adult cochlea. This highlights the need for further optimization of these protocols, but also the possibility of using cochlear organoids for screening and identification of factors that extend the time window permissive for regeneration.

The capacity and efficiency of generating organoids is often used as a read-out for the presence and activity of tissue-specific progenitors, whether they are active participants in cellular turnover or quiescent until recruited for repair. For example, for some tissues,

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such as the intestine, the stem cell pool actively turns over *in vivo* (Sato et al., 2009), whereas for others, such as the lung, the cells are replaced less rapidly (Lee et al., 2017; Zacharias et al., 2018). Nevertheless, they can both give rise to organoids when stimulated by Wnt signaling. Interestingly, this technology has been useful for the identification of alveolar and bronchial stem cell compartments in the lung (Lee et al., 2017; Zacharias et al., 2018). These authors concluded that Axin2-expressing alveolar progenitors are facultative stem cells that do not participate in normal homeostasis, but are recruited upon damage (Zacharias et al., 2018). This is closely analogous to the data obtained with cochlear organoids; despite their postmitotic state and low activity in tissue repair, Lgr5-positive supporting cells can act as facultative hair cell progenitors as shown by lineage tracing experiments (Shi et al., 2013, 2012). Lgr5 upregulation and an increase in Wnt responsiveness may induce a progenitor identity in supporting cells that are normally quiescent.

The culture conditions developed by McLean et al. (2017) do not attempt to recreate the endogenous relationships between the cell types of the organ, but, rather, produce a high yield of a single cell type by maximizing the expansion of Lgr5-positive progenitors in a first step, and subsequently their conversion to hair cells. When left to differentiate spontaneously, however, both supporting cells and hair cells are formed, recapitulating the cellular composition of the sensory epithelium from which they were derived (Roccio et al., 2018).

Cochlear organoids from the human fetal prosensory domain

Human tissue-specific hair cell progenitors have so far only been expanded from the fetal cochlea and have been shown to express p27, SOX2, LGR5 and p75, as in mice (Roccio et al., 2018). Although LGR5 is expressed at the mRNA level in the developing prosensory domain, the antibody tools currently available have not allowed exploitation of this marker for cell isolation. Instead, the authors have used a combination of the surface markers EPCAM and CD271 (NGFR/p75) to isolate these cells, with the epithelial marker EPCAM allowing for the isolation of all cochlear duct resident cells, and CD271 marking the prosensory domain region (Fig. 2B). In the presence of growth factors and a 3D ECM scaffold provided by Matrigel, these cells, which are already postmitotic *in vivo*, regain proliferative activity, expand and display epithelial organization and polarity. Because cells were isolated from fetal samples before the appearance of hair cells, *in vitro* differentiation in this system represents a normal developmental process and organoids contain both sensory hair cells and supporting cells after several weeks in culture. *In vitro*-differentiated hair cells display espin and F-actin-positive stereociliary bundles and take up aminoglycosides, such as Texas Red-conjugated gentamycin, indicating their potential use to screen for ototoxicity and regeneration *in vitro* (Fig. 2D). Although the fetal study has relied on samples of weeks 10/11 of development, tissue-specific progenitors may already be fate-committed, even at these early stages, thus limiting their capacity for cell expansion and differentiation. Treatment with chromatin modifiers with the aim of de-differentiating the tissue-specific progenitors could improve differentiation efficiency, as previously shown for the postnatal murine tissue.

Although seemingly similar responses to pathway modulation between mouse and human tissue-specific progenitors have been observed, such as their response to Wnt activation and Notch inhibition, potential differences should be investigated in detail. This will enhance translation of therapeutic strategies targeting *in situ* progenitors.

Inner ear organoids from pluripotent stem cells

In vitro guided organogenesis in 3D culture

Several studies have derived sensory hair cell-like cells *in vitro* by differentiating murine embryonic stem cells (mESCs) and murine induced pluripotent stem cells (mIPSCs) (Oshima et al., 2010) as well as human embryonic stem cells (hESCs) (Chen et al., 2012; Ealy et al., 2016; Ronaghi et al., 2014) in 2D culture. Similar approaches have been undertaken for the generation of otic sensory neurons (Chen et al., 2012; Corrales et al., 2006; Matsuoka et al., 2017; Shi et al., 2007). Different degrees of maturation have been obtained in these studies, but the yield of terminally differentiated cells has been limited. Common to all these approaches is directed differentiation: an attempt to guide pluripotent cells through stages of normal otic development, using growth factors and small molecules, experimentally validated by characterization of the intermediate steps of lineage progression. More recently, direct reprogramming strategies, based on transcription factor overexpression, have proved effective for neuronal differentiation (Noda et al., 2018; Rivetti di Val Cervo et al., 2017) and have also been exploited for *in vitro* derivation of hair cells (Costa et al., 2015).

A major advance for *in vitro* guided organogenesis for the generation of inner ear sensory cell types has come about with the development of protocols that combine initial patterning with the self-organization properties of pluripotent stem cells in 3D cultures. This approach has been applied successfully for the generation of neural tissue (Eiraku et al., 2008) and retinal tissues (Eiraku et al., 2011; Nakano et al., 2012), and involved initial patterning to neural ectoderm followed by spontaneous differentiation of multiple cell types and self-organization in tissue-like structures. The same protocols have been exploited for the generation of 'brain organoids' (Hattori, 2014; Lancaster et al., 2013; Quadrato et al., 2017). These protocols have now been further refined by implementation of patterning approaches to generate specific cellular fates and dorsal/ventral identities (Brown et al., 2018; Cederquist et al., 2019; Qian et al., 2016; Sakaguchi et al., 2015). In all cases, multiple cell types of the specific organ of interest could be generated *in vitro* and displayed remarkable similarities with their physiological counterparts in terms of tissue architecture, as well as transcriptional profiles (Camp et al., 2015).

Using a combination of guided differentiation and spontaneous self-organization in 3D cultures, Koehler and colleagues have succeeded in generating otic vesicle-like structures containing functionally mature sensory hair cells from mESCs (Koehler and Hashino, 2014; Koehler et al., 2013). Initial steps for definitive ectoderm induction using serum free quick aggregation methods in the presence of Matrigel were followed by the induction of non-neural ectoderm, using transient exposure of the cells to BMP. The tissue was then coaxed to differentiate to a placodal fate by downregulation of BMP signaling and stimulation of FGF signaling, based on the known role of FGF in otic fate specification in the embryo (Litsiou et al., 2005; Martin and Groves, 2006). Subsequent studies from the same group have optimized the protocol by inhibiting GSK3β, using CHIR99021 to activate Wnt signaling and to increase otic fate induction (DeJonge et al., 2016; Liu et al., 2016) (Fig. 3A). The same protocol has been successfully translated to hESCs and human induced pluripotent stem cells (hIPSCs), by modifying the timing to match human fetal development (Jeong et al., 2018; Koehler et al., 2017; Munnamalai and Fekete, 2017). While in the murine system the first hair cells appear at 2-3 weeks (15-21 days) *in vitro*, differentiation is extended to 10 weeks (70 days) for human cells (Fig. 3).

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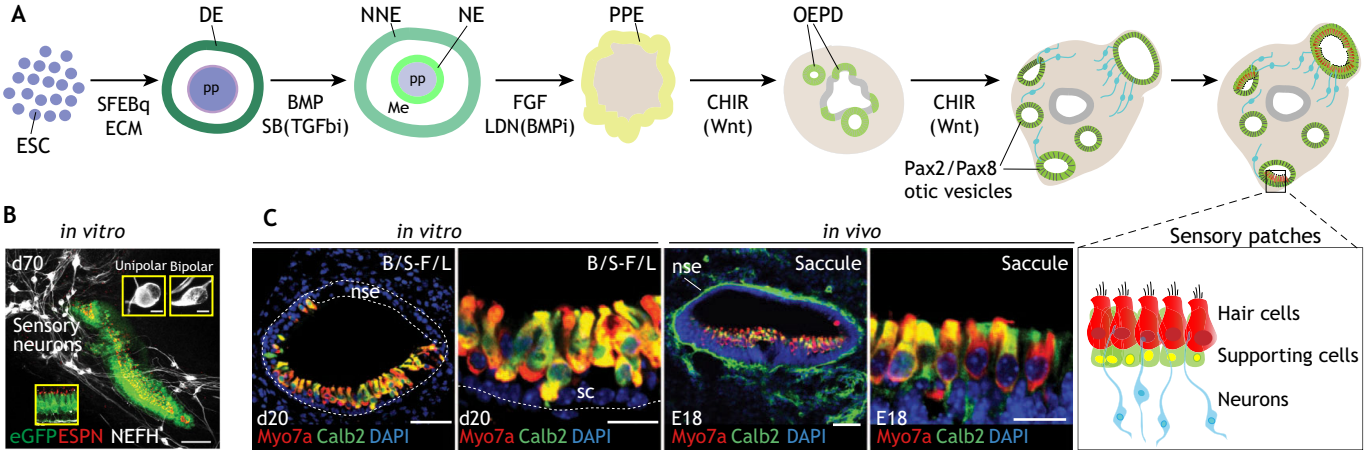

Fig. 3. Pluripotent stem cell-derived inner ear organoids. (A). Schematic of *in vitro* guided organogenesis from pluripotent stem cells to otic tissue adapted from the mouse and human protocols. Embryonic stem cells (ESC) are aggregated with serum-free embryoid body-quick (SFEBq) methods and embedded in Matrigel (ECM) to induce definitive ectoderm (DE) at the surface of the aggregate. Non-neural ectoderm (NNE) is induced by BMP4 addition (or its endogenous production) and concomitant inhibition of mesendoderm fate with a TGFβ inhibitor (SB; SB451542). Pre-placodal ectoderm (PPE) fate is promoted by the addition of FGF (Fgf2) and BMP inhibitor (LDN; LDN193189). Otic epibranchial placode (OEPD) and otic fate are promoted by activation of Wnt signaling with GSK3β inhibitor, CHIR99021 (CHIR). Pax2/Pax8 otic vesicles form within the aggregate and further develop into Jag1+/Sox2+ prosensory patches (not shown) and later into sensory patches containing hair cells (red) and supporting cells (green), as well as otic sensory neurons (blue). (B) Representative images from Koehler et al. (2017) showing inner ear organoids derived from the differentiation of hESCs. The mechanosensitive hair cells are labeled by Atoh-1 GFP (green), hair bundles are stained for espin (red) and co-differentiated sensory neurons are shown in white (NEFH) at day 70 (d70) of *in vitro* differentiation. (C) Comparison of *in vitro*-generated sensory hair cells (20 days *in vitro*; d20) from mESCs and hair cells resident in the murine saccule at E18 of mouse embryonic development. Hair cells are immunostained for Myo7a (red) and calbindin 2 (calb2, green). Figures from Koehler et al. (2013). Dotted lines delineate the border of the vesicle and the non-sensory epithelium (nse). B/S, BMP/SB; F/L, FGF/LDN; Me, mesoderm; NE, neural ectoderm; pp, pluripotent; sc, supporting cells. Scale bars: 100 µm in B (5 µm in inserts); 50 µm in C (1st and 3rd panels); 25 µm in C (2nd and 4th panels).

Characterization of PSC-derived inner ear organoids

Hair cells generated by these means are organized in patches, surrounded by supporting cells, in vesicular structures that resembled the sensory patches of the vestibular organs. Electrophysiological characterization of the cells, bundle morphology and synaptic connections have led to the conclusion that the derived cell types resemble vestibular rather than cochlear hair cells (Koehler et al., 2017; Liu et al., 2016) (Fig. 3B,C). Mechanosensory hair cells in the vestibular organs are present by week 10 of human development, and by week 12 form hair bundles, whereas cochlear hair cells only differentiate starting from week 12-14 (Locher et al., 2013; Roccio et al., 2018). Whether prolonged culture time would lead to generation of additional hair cell phenotypes needs to be explored. Additional factors may be missing for the specification of cochlear fate. For example, sonic hedgehog (SHH) signaling has been suggested to induce ventral identity in the otic vesicle (Bok et al., 2007; Riccomagno et al., 2005) but is missing from current differentiation protocols. Direct comparison of the human tissue-specific progenitor population and human PSC-derived cell types by single cell analysis is required to assess the fidelity of *in vitro* otic development.

The culture conditions developed by Koehler and colleagues also give rise to bipolar neurons that form synaptic contacts with the newly generated hair cells (Fig. 3B). The common developmental origin of sensory neurons and sensory epithelia, and the earlier differentiation of otic neurons compared with hair cells during inner ear development, suggests co-induction of this cellular fate in the organoid cultures (Koehler et al., 2013, 2017). Plating of the organoids on Matrigel during otic placode induction has allowed characterization of these neurons (Perny et al., 2017). The analysis of gene expression at different time points during the induction protocol using mESCs has confirmed that cells transit through stages of otic development and neuroblast specification, finally giving rise to mature neurons expressing markers of spiral and vestibular ganglion neurons.

Future directions for drug screening and disease modeling

Promise of inner ear organoids

The possibility of generating human sensory cell types *in vitro* opens the door to the development of novel therapeutic strategies for hearing loss (Géléoc and Holt, 2014). Stem cell-derived sensory cells allow for testing drug sensitivity or toxicity and for validating gene therapy approaches. They also represent a source of cells for cell replacement strategies. Moreover, they are an alternative tool for studying otic development *ex vivo* to gain insight into the consequences of genetic mutations on inner ear development and the functional differentiation of human hair cells, which are otherwise highly inaccessible.

Until recently, obtaining human hair cells in a dish was not possible. Although fetal tissue is an option for this type of analysis, restricted access to the material and the associated ethical concerns pose obstacles. In addition, the variability in developmental stage at the time of tissue collection, the variability in tissue integrity and the immature stage of the organ hinders their use for screening purposes. Nevertheless, proper implementation of culture conditions, using the organoid cultures discussed above (Roccio et al., 2018), could develop this cell source into a suitable platform for drug screening or validation. Specifically, tissue-specific progenitors could be expanded using suitable culture methods in order to obtain large number of cells *in vitro* – independently of tissue donation – and these could be subsequently differentiated to sensory neurons or hair cells. The possibility of optimizing strategies and protocols in the tissue-specific murine progenitors offers a platform for assessing gene modifications, small molecule perturbations and culture conditions that can then be translated to human progenitor cells.

Pluripotent stem cell-derived otic cells have the advantage that there are potentially no limitations on their availability or scalability. However, the field is still in its infancy and ‘proof-of-concept’ differentiation assays need to be translated to robust, reproducible and

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efficient protocols to make these tools suitable for drug or genetic screening. As discussed above, the best results to date, in terms of yield and cellular function of the generated cell types, in particular for hair cells, have been obtained in 3D organoid cultures. Despite the fact that the induction of otic placode fate is efficient, only a fraction of the inner ear organoids contains functional sensory hair cells. It is less clear what drives the differences in outcomes in these protocols (Koehler et al., 2017). The generation of reporter lines to track lineage differentiation would lead to improved protocols, with higher efficiency and yield (Hartman et al., 2018; Koehler et al., 2017; Schaefer et al., 2018). The rapidly evolving field of gene editing will surely lead to further implementation of these tools (Nie and Hashino, 2017). In addition, optimization and standardization of the culture conditions may improve reproducibility, for example by employing automated liquid handling robots.

Drug screens

Drug screening of toxic, protective or regenerative compounds is generally carried out *ex vivo*, using the micro-dissected sensory epithelium of young postnatal rodents. However, the incomplete differentiation of the organ of Corti at early stages can result in a response that differs between young postnatal animals and adults, because of the higher regenerative potential and differences in sensitivity of neonates to ototoxic agents (Henley and Rybak, 1995). Alternatively, toxicity and regeneration can be studied directly *in vivo*, using animal models in which hearing thresholds or sensory cell survival can be tested (Abbas and Rivolta, 2015; Abbas and Rivolta, 2019; Breglio et al., 2017; Furman et al., 2013; Kujawa and Liberman, 2019). The level of complexity of animal testing using rodents is substantial, and as only a small number of compounds or concentrations can be handled simultaneously, these experiments are difficult to scale up to medium- or high-throughput screens. A higher throughput pipeline utilizes the zebrafish larva neuromast to study hair cell damage, protection and regeneration (Chiu et al., 2008; Stawicki et al., 2015). Large libraries of compounds can be tested in these models and 'hit' compounds can be re-validated in rodents (Kenyon et al., 2017; Ou et al., 2009; Owens et al., 2008). However, the transferability of the findings obtained in rodents or non-mammalian vertebrates to humans is uncertain.

Human sensory cells derived from pluripotent stem cells offer a new alternative to these methods; however, organoid cultures also have limitations. The embedding of 3D otic vesicles in large organoids can limit drug penetration. In addition, drug exposure may not mimic the physiological situation, because the drug may reach the cells through their baso-lateral membranes rather than their apical domains. Finally, the large dimensions of the organoids, which reach 1-2 mm in diameter, requires a specific imaging set up for assessing differentiation efficiency in whole-mount culture. Confocal, multiphoton or light-sheet imaging pipelines need to be developed in parallel to match the throughput of the assay (Rios and Clevers, 2018). Although 3D inner ear organoid cultures have advanced the field by providing unprecedented cellular maturation, alternative solutions that rely on bioengineering strategies and organ-on-chip technology may provide novel ways to culture and differentiate these cells. This can be achieved by providing the correct tissue stiffness, cell-cell contact and flow conditions to improve tissue accessibility and encourage maturation, both for drug exposure and image-based analysis (Ronaldson-Bouchard and Vunjak-Novakovic, 2018; Rossi et al., 2018).

Organoids derived from murine cochlear tissue-specific progenitors also provide a new tool to study toxicity and regeneration (McLean et al., 2017). We have, for example, transduced the Lgr5-positive cells with viruses to perform CRISPR/Cas9 gene silencing, as well as performing drug screening to increase progenitor expansion and hair cell differentiation (Lenz et al., 2019). Modulation of the EGF receptor family member ErbB2, a known regulator of cell cycle progression in different tissues, through small molecules, in combination with activation of Wnt signaling, results in the expansion of the Lgr5-positive population in the organoid cultures (Lenz et al., 2019). This finding is consistent with *in vivo* evidence, which shows that activation of the ErbB pathway through chemical or genetic means induces proliferation of supporting cells and generation of supernumerary hair cells (Zhang et al., 2018). Current strategies converting cochlear progenitors to hair cells through activation of Wnt signaling and inhibition of Notch signaling have led to the generation of remarkable numbers of hair cells that display stereociliary bundle morphology and functional properties of mature hair cells. They further express markers of cochlear hair cells, including IHCs and OHCs, whereas generation of cochlear hair cells from ESCs and IPSCs has been difficult to achieve (Koehler et al., 2017; Liu et al., 2016). Derivation of the specialized cell types, in addition to their smaller dimensions and more uniform cellular composition compared with PSC-derived inner ear organoids, make them likely to be more suitable for ototoxicity screens.

Genetic screens

Inner ear organoids provide a model for the study of genetic defects that cause hearing loss. Compared with *in vivo* mouse models with hearing deficits, the organoids offer a faster means to gain an understanding of the molecular consequences of mutations. Indeed, inner ear organoids derived from hESCs and hIPSCs are particularly suitable for the study of genetic defects associated with peripheral sensory cells. Patient-derived hIPSCs or gene-edited lines can be used to study developmental defects. They are also an ideal tool to assess restoration of function and to validate gene therapy approaches after correction in the organoid cultures. In fact, they allow the testing of different viral vectors (Pan et al., 2017; Suzuki et al., 2017; Wang et al., 2018) or delivery strategies of molecular components required for gene replacement or gene correction (Gao et al., 2018; Lentz et al., 2013; Rees et al., 2017; Yeh et al., 2018) in human sensory cells, which is necessary for clinical applications. To this end, robust differentiation protocols, in terms of efficiency and maturation of the cells, will need to be established in order to validate the changes in phenotype caused by a genetic defect (Brown et al., 2008).

A demonstration of such an approach has been recently published using inner ear organoids derived from mESCs (Tang et al., 2019). Here, the functional consequences of mutations in the transmembrane protease TMPRSS3 (associated with hearing loss in humans) were analyzed. Although hair cells developed properly in culture, early signs of hair cell degeneration could be detected in the mutant lines.

Optimization of culture maintenance and extended culture periods may be needed to identify functional deficits associated with 'late' phenotypes, for example hair cell degeneration resulting from defects in stereocilia organization as seen in Usher syndrome (Emptoz et al., 2017; Pan et al., 2017). Although this review has focused on the generation of sensory cell types from pluripotent stem cells, additional cellular populations could be generated *in vitro*, to allow characterization of genetic defects affecting other components of the inner ear. For example, plating the outer epithelium and otic vesicle derived as in Koehler et al. (2013) on 2D substrates or feeder layers has allowed the generation of supporting cell-like cells in which to study mutations in connexin 26, encoded by the *GJB2* gene (Fukunaga et al., 2016), that accounts for a large proportion of hereditary deafness.

Cell replacement

Cell replacement strategies, relying on transplantation of *in vitro*-derived sensory cells (e.g. from ESCs or IPSCs) have been considered in recent years as a therapeutic option for hearing loss. Some effort has been devoted to infusion of hair cells or their progenitors into the cochlea (Beisel et al., 2008; Lopez-Juarez et al., 2019). In addition, engraftment of cells into the sensory epithelium has been reported (Lopez-Juarez et al., 2019). However, hair cell replacement has been considered an unlikely strategy because of the complex architecture of the sensory epithelium and difficult surgical access. Therefore, sensory cell types generated through organoid technology, or alternative methods, appear to be more suitable for *in vitro* screening of compounds that trigger endogenous regeneration, as discussed above.

Transplantation of PSC-derived (otic) neuronal progenitor cells into the modiolus/nerve trunk has been advancing in preclinical models and is a viable option to repopulate the spiral ganglion after cell loss resulting from neuropathies (Chen et al., 2012; Corrales et al., 2006; Shi et al., 2007). This approach could increase the effectiveness of neuroprosthetic stimulation in cochlear implant recipients (Abbas and Rivolta, 2019). Whether spiral ganglion neuron progenitors derived using the latest 3D induction protocol would lead to improved functional outcomes remains to be tested.

Conclusion

The rapid technological advancements in stem cell technologies, organoid culture, genome editing, gene therapy and single cell analysis provide unique and unprecedented opportunities to model diseases and develop personalized therapies for hearing loss. The generation of human sensory cells using inner ear organoids from pluripotent stem cells, represents an exciting new tool to study developmental processes and dysfunction and to validate therapeutic approaches, such as chemical-pathway modulation, gene correction and gene therapy. Benchmarking of these *in vitro*-generated cell types to tissue-specific human and murine progenitors, using single cell transcriptional profiling, will provide solid evidence of the similarities between the *in vitro*-derived cell types and their *in vivo* counterparts, and at the same time allow the optimization of differentiation protocols. Organoid cultures of tissue-specific cochlear progenitors also provide a tool to study strategies for tissue regeneration. Pathways identified as promoting organoid generation or differentiation *ex vivo* could be targeted chemically or genetically *in situ* and combined with mouse models to study hearing loss and regeneration, lending further confidence to their clinical translation.

Competing interests

The authors declare no competing or financial interests.

Funding

This work was supported by the National Institutes of Health (grant DC14089) and by the European Union Seventh Framework Programme (grant 603029). Deposited in PMC for release after 12 months.

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